LIBRARY

JUL 15 1929

VOLUME IV NUMBER 3

STAIN TECHNOLOGY

JULY, 1929

CONTENTS

Progress in the Standardization of Stains
Safranin. H. J. CONN 65

Influence of Impurities upon Dyes. H. J. CONN 68

Stains Recently Certified 70

Announcement 71

Stain Solubilities. Part IV. W. C. HOLMES 73

The Mechanism of Staining. W. C. HOLMES 75

Detailed Differentiation of Bacteria by Means of a Mixture of Acid and Basic Dyes at Different pH-values. ALEXANDER V. TOLSTOOUHOV 81

A Note on the Gram Stain. ALEXANDER ZEISSIG 91

Laboratory Hints from the Literature 93

Published by the

COMMISSION ON STANDARDIZATION OF BIOLOGICAL STAINS

Entered as second-class matter at the Post Office, Geneva, N. Y.

STAIN TECHNOLOGY

ISSUED QUARTERLY SUBSCRIPTION PRICE $1.50 PER YEAR

Editor and Business Manager
H. J. CONN
Soil Bacteriologist, N. Y. Agr. Exp. Station

Consulting Editors
CHEMISTRY: W. C. HOLMES Color Lab., U. S. Dept. of Agriculture
ZOOLOGY: S. I. KORNHAUSER School of Medicine, Univ. of Louisville
PATHOLOGY: F. B. MALLORY Boston City Hospital
BOTANY: W. R. TAYLOR Univ. of Pennsylvania

SUGGESTIONS TO AUTHORS SUBMITTING MANUSCRIPT

Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Agr. Exp. Station, Geneva, N. Y. Articles to be accepted for publication must ordinarily deal with the nature or uses of biological stains. Articles dealing with microscopic technic in general, however, will be accepted in most cases, if they have a close bearing on staining procedures. Brief notes concerning new staining methods are especially solicited. It is understood that articles submitted for publication have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

To be accepted for publication, manuscript should be original typewritten copy (not carbon copy) either double or triple spaced, with wide margins. Special attention hould be given to the headings of tables and to legends for illustrations. These in all cases should be complete in themselves, so as to make the table intelligible to anyone studying it, without consulting the text of the article. In the case of complicated tables, typewritten manuscript is not absolutely required, provided all words and figures are carefully written or printed in a legible hand. All references to literature must be carefully verified and in ordinary cases the complete title and reference should be given.

Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which can be used as an author's abstract by abstracting journals. This summary must therefore be complete in itself, and must contain all the important facts brought out in the paper.

All illustrations should accompany the manuscript and should be numbered with the legend for each written on separate paper and attached loosely to the illustration. They should always be referred to in the text. Line drawings must be made in India ink. Photographs for half-tones must be on paper giving black and white tones and should preferably have a glossy finish.

Authors are especially urged to make all staining formulae definite and accurate. If known to the author, the dye content of the stain should be given and the exact dye used should be specified. If this information is not known, the name of the manufacturer should be stated and that of the stain itself exactly as worded on the label. Certified stains should be specified by certification number.

STAIN TECHNOLOGY

VOLUME IV JULY, 1929 NUMBER 3

PROGRESS IN STANDARDIZATION OF STAINS

SAFRANIN

We have at hand a catalog of Grübler & Co. of uncertain date, but issued without question before the war. In this catalog the following types of safranin are listed:

Safranin nach Curtis
" O, wasserloslich, Pfitzner
" Flemming
" rein (Phenosafranin)
" spirituslöslich

These same types of safranin are also given in the 1928 price list of this same company with one additional grade, namely safranin yellow water soluble.

In other words the German concern lists a greater variety of types of safranin now than they did before the war, while the American stain companies, largely at the recommendation of the Stain Commission, are carrying only one regular biological grade, namely safranin O. This naturally raises the question whether there is any need of more than one type of safranin for the biologist, or whether his wants are just as well supplied with the single grade furnished by the American concerns. In the latter case, the American plan of standardizing on one type is the better, both for the dealer and the consumer. It prevents the necessity of carrying more than one product in stock, and thus lowers expenses; it avoids confusion on the part of the customer and the possibility that he has to order two or more different samples before securing the grade he requires.

To answer this question it is necessary to consider the purposes for which safranin is employed. The Stain Commission has always regarded the most important use of safranin to be for cytological work. This is not because more safranin is necessarily purchased for this purpose than for any other, but because the requirements of a good cytological safranin are much more severe than for a safranin intended for any other biological technic. It is used sometimes, it is true, as a general nuclear stain in histological work, but there does not seem to be much difficulty in finding a safranin satisfactory for such purposes. It is also employed by bacteriologists as a counter-

stain in the Gram technic, but here again almost any safranin that has been tried gives good results in this procedure. From cytologists, however, complaints in regard to this dye have been numerous.

In cytology it is employed as a chromatin stain, sometimes in contrast to light green as a cytoplasm stain, at other times with gentian violet for staining the nuclear structures other than chromatin; or again in the Flemming triple stain where it must contrast both with a violet nuclear dye and orange G in the cytoplasm. For such purposes as these safranin has always been recognized as a rather tricky stain to handle. It may not stain sufficiently intensively, it may be too yellow to contrast with the orange, or too nearly purple to contrast with the violet. Cytologists have recognized for a long time that different batches of safranin might not be equally satisfactory, and have not understood how to avoid inconsistent results.

For this reason the efforts of the Stain Commission in standardizing safranin have been to secure a reliable product for cytological work with the expectation that a sample satisfactory for this delicate procedure would also work in other technics. This has proved to be the case, and for this reason it is believed that one grade is sufficient for the use of biologists. It is felt indeed that some of the inconsistencies in the early results with this dye may have been caused by the very fact that the purchaser was confused in ordering, due to the multiplicity of types listed in catalogs.

The type of safranin which seems to be most generally satisfactory is that known as safranin O. It is characterized by a spectrophotometric curve having its absorption maximum at about 515 mμ. Of those safranins listed in the Grübler catalog, safranin pure (rein) is a bluer shade than this and safranin yellow water soluble is a more yellow shade. No special purpose has yet been found for which these two shades are more satisfactory. Certainly the former is unsatisfactory if used in contrast to a violet dye, the latter if employed with an orange stain. The two types of safranin sold by the Grübler company designated with the names of Curtis and Flemming respectively have not yet been investigated. The other two types, however, denoted water soluble and alcohol soluble respectively, have been studied and both seem to be identical. These two products agree so far as we can tell with safranin O and both are equally soluble in water and in alcohol.

In connection with the water and alcohol soluble types of safranin, it is interesting to note that Chamberlain[1] has called for a mixture

[1]Chamberlain, C. J. Methods in Plant Histology. *4th Ed.*, Chicago, 1924. See p. 64.

of the two in the safranin solution which he employs in the Flemming triple stain. This formula of Chamberlain's was based upon prewar dyes and it is entirely possible there may have been a difference between the water soluble and alcohol soluble safranins on the market at that time. It certainly seems, however, to be impossible to find any differences in the case of the two products now available. For this reason there seems to be no present reason for following the directions given by Chamberlain for mixing these two types of safranin. One should rather employ a safranin known to give good results in the technic in question, and make no attempt to mix two different types as called for by Chamberlain.

The probable explanation of Chamberlain's better success in this procedure when mixing the alcohol soluble and water soluble safranin is that in the case of a dye showing so much difference between different lots in the matter of behavior, more uniform results would be obtained with a mixture of two lots than with a single batch. It is unfortunately the case that not all batches of safranin O give the same results. This fact was recently emphasized by Miss Haynes[2] who showed the existence of quick acting and slow acting safranins. Either of these types may be used in the Flemming triple stain, but with one the staining must be prolonged several hours, with the other it may be finished in a comparatively few minutes.

Altho the Stain Commission recognizes the distinction between these two types of safranin, the explanation of their different behavior has not yet been obtained. The dye itself in these two types seems to be the same, so far as can be recognized by tests known at the present time. One company in fact has submitted several batches of safranin all prepared from the same lot of crude dye, some of which have been rapid in their action, others slow. These facts certainly suggest that the difference between these two types of safranin is not in the dye itself, but in the impurities that may be present. No actual correlation, however, with either the ash content or the hydrogen-ion concentration of the sample has yet been established. Meanwhile the user of safranin should take into account the fact that there are these two types on the market. In selecting the technic to employ, one should use longer or shorter periods of staining, according to which type of safranin is at hand. If anyone obtains poor results with a safranin that has been approved by the Commission, we can easily inform the user of a satisfactory technic for the purpose in question, using his particular batch, if he will let us know its certification number. Both types of safranin have been certified in the past; but on account of the manifest superiority of

[2]Haynes, Rachel. Types of safranin and their use. Stain Techn. 3, 43. 1928.

the more rapid technic when it is possible to employ it, preference is now being given to the more quickly acting safranin. Those safranins certified in the future will probably all be of the more rapidly acting type.

Influence of Impurities Upon Dyes

Too much emphasis cannot be laid upon the influence of impurities on the action of a stain. This action may be either harmful or beneficial. When the production of stains was begun in the United States, it was assumed at first that the action of any impurity would be harmful. When a user, therefore, obtained unsatisfactory results with a dye, he was likely to complain to the manufacturer or dealer that it was not sufficiently pure. There was accordingly a strong demand for "pure" dyes which some of the stain companies did their best to meet so far as it was possible to do so. As a result the American dyes at present on the market are noticeably higher in dye content than those sold before the war. This represents in general a very marked improvement, altho there is at least a possibility that in some instances purification has been carried a great deal further than necessary.

There are two ways in which impurities may come to be present in a dye. In the first place, a strictly pure dye is impossible because in the course of manufacture, certain mineral salts are either formed or introduced, and cannot be completely eliminated by any method of purification. A dye content of 90% is about the best that can be hoped for in the case of any dye, and even this degree of purity represents an expense that may perhaps seldom be justified. In the second place, an impurity is often intentionally added to a dye so as to standardize its strength. This is most commonly done in the case of dyes employed in the industry, but it may sometimes be done also in the case of biological stains. When adding an impurity intentionally in order to dilute a dye, the manufacturer selects a supposedly inert ingredient. The diluents most commonly employed in this way are dextrin and sodium chloride. Whether or not these substances are actually inert has not yet been determined.

There are various ways in which an impurity may influence the action of a stain. If it changes its reaction, it will have a pronounced effect, as basic dyes stain more readily at an alkaline reaction, acid dyes at an acid reaction. Even a neutral salt may greatly alter the behavior of a stain, either because it changes the solubility of the dye, or else because the presence of the salt alters the rate at which the dyes will be adsorbed by the tissue.

One of the most striking illustrations of this salt effect has been brought out recently by Conn and Holmes[3] in the case of rose bengal and related dyes. This action was first encountered when it was discovered that a sample of rose bengal not staining sufficiently intensely in distilled water, would give good results when made up in tap water. This phenomenon was apparently due to the presence of calcium or magnesium salts in the tap water, probably calcium. By increasing the amount of calcium present—to a concentration in fact no greater than sometimes found in a hard tap water—altogether too intense staining was obtained.

This has naturally suggested that when different samples of dye vary in the intensity of their staining action as in the case of safranin discussed above, the cause may be found in variations of the impurities present. Altho this has not yet been proved, there is very good evidence that one can alter the staining intensity of a dye sample by adding certain salts to the staining fluid. This procedure is suggested when anyone obtains poor staining effects with some particular sample of the dye. One may sometimes employ to best advantage an inorganic salt as in the case of rose bengal, or at other times a mineral salt of an organic acid as when ammonium oxalate is used with crystal violet in the Gram stain. As yet no rules can be laid down to govern the choice; but the mere knowledge that such an influence is possible should in any case be of help to a clever technician in adapting stains to some use for which they do not at first seem satisfactory.

H. J. Conn

---

[3]Conn, H. J. and Holmes, W. C. Certain factors influencing the staining properties of fluorescein derivatives. Stain Techn. **3**, 94–104. 1928.

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the April number of this journal.

Stains Certified February 15 to May 15, 1929*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Methylene blue (medicinal)	CA 8	85%	As bacteriological and histological stain	Feb. 15, 1929
Methylene blue (medicinal)	NA 4	87%	As bacteriological and histological stain	Feb. 15, 1929
Gentian violet	CB 7	78%	As bacteriological and cytological stain	Mar. 7, 1929
Methyl violet 2B	CMv 2	81%	As bacteriological and cytological stain	Mar. 7, 1929
Methyl green	NG 4	55%	For histological use and as constituent of Pappenheim stain	Mar. 7, 1929
Thionin	NT 8	90%	As nuclear stain for frozen or paraffin-fixed tissue	Mar. 7, 1929
Fuchsin, basic	NF 14	78%	As bacteriological stain, and for use in Endo medium	Apr. 2, 1929
Brilliant cresyl blue	NV 6	66%	As vital blood stain	Apr. 16, 1929
Fuchsin, acid‡	NR 4	57%	As histological stain and for use in Andrade indicator	May 10, 1929
Indigo carmin	CI 2	——	As histological stain	Apr. 27, 1929
Crystal violet	NC 7	90%	As bacteriological and histological stain	May 3, 1929
Wright's stain	NWr 5	——	As blood stain	May 11, 1929
Phloxine	NPh 3	87%	As histological stain	May 14, 1929

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

‡Certified provisionally pending investigations by the company as to the manufacture of acid fuchsin by the methods of Scanlan, French, and Homes.

ANNOUNCEMENT

At a meeting of the Executive Committee of the Stain Commission, held at Rochester, N. Y., March 30, 1929, the desirability of increasing the size of the Executive Committee was discussed, and the following amendment to the Constitution was approved for submission to the members of the Commission.

AMENDMENT TO THE CONSTITUTION

EXECUTIVE COMMITTEE. Beginning with May 1, 1929 the Executive Committee shall not be limited to five members as specified in Article IV, but may be increased in size to a membership of not more than eight. The candidates for membership shall be the official Society Representatives on the Commission. The new members shall be elected by a majority vote of the existing Executive Committee, the vote to be taken either by mail or at one of the meetings of said committee. The same method of election shall be followed in the case of any vacancy on the Executive Committee, due to resignation or other cause.

This amendment was submitted by mail to the members of the Commission on April 20th, with a ballot form enclosed. Up to the date of writing, 35 votes in favor of the amendment have been received. As this is distinctly more than a majority of the entire membership, the amendment has carried and is now in effect.

Acting on the authority thus given them, the five members of the Executive Committee before the adoption of this amendment have voted for the following as additional members of the Committee:

J. W. Churchman, Cornell Medical School, N. Y. City; representative of the American Medical Association.

W. C. Holmes, Color and Farm Waste Division, Department of Agriculture, Washington, D. C.; representative of the American Chemical Society.

STAIN SOLUBILITIES

Part IV[1]

W. C. Holmes

(Contribution No. 164 from Color and Farm Waste Division, Bureau of Chemistry and Soils, Department of Agriculture, Washington, D. C.)

Further solubility data on dyes are recorded in Table 1. Except when otherwise specified the values reported are expressed as grams of anhydrous dye per hundred cubic centimeters of saturated solution.

Many of the values recorded have reference to the solubilities of various salts of fluorescein and of its halogenated derivatives. Certain statements made previously elsewhere[2] respecting the relative aqueous solubilities of some of these compounds, which were based upon mere visual observation and for which the writer was responsible, have now been shown to be partly erroneous. Salts of erythrosin are less soluble than the corresponding salts of eosin, as was anticipated, but salts of phloxin are more soluble rather than less soluble than those of eosin, and salts of rose bengal are more soluble rather than less soluble than those of erythrosin. The data afford interesting evidence on the influence of halogen substitution on solubility with this class of dyes altho extended generalizations would only be warranted by a more comprehensive investigation. Evidently both the nature of the substituent and the location of the substitution affect solubility. Solubility is decreased by substitution within the resorcinol residues of the dye and is decreased more by iodine than by bromine. On the other hand substitution in the phthalic anhydride residue increases solubility.

The solubility of the sodium, magnesium, calcium, and barium salts of these dyes was found to decrease, in general, in the order given, as was anticipated.

Certain of the data obtained with these salts appear distinctly anomalous and may possibly have been affected by hydrolysis or some other factor.

---

[1]The previous papers of this series have appeared in Stain Techn. 2, 44–49, 68–70, 1927; 3, 12–13, 1928.

[2]Conn, H. J. and Holmes, W. C. Certain Factors Influencing the Staining Properties of Fluorescein Derivatives. Stain Techn. 3, 94–104, 1928.

TABLE 1. DYE SOLUBILITIES AT 26°C.

	Water	95% Alcohol
Victoria Yellow; C. I. 8	1.66	1.18
Martius Yellow (sodium salt); C. I. 9	4.57	0.16
Ponceau 2 G; C. I. 28	1.75	0.21
Erika B; C. I. 130	0.64	0.17
Janus Green; C. I. 133	5.18	1.12
Azo Acid Yellow; C. I. 146	2.17	0.81
Brilliant Croceine; C. I. 252	5.04	0.06
Erythrin X; C. I. 254	6.41	0.06
Victoria Green 3 B; C. I. 659	0.04	2.24
Guinea Green B; C. I. 666	28.40*	7.30
Light Green SF Yellowish; C. I. 670	20.35	0.82
Fast Green FCF, (*p*-hydroxy derivative of C. I. 671)	16.04	0.35
Victoria Blue 4 R; C. I. 690	3.23	20.49
Patent Blue A; C. I. 714	8.40	5.23
Cyanole extra; C. I. 715	1.38	0.44
New Victoria Blue R; C. I. 728	0.54	3.98
Pyronin G; C. I. 739	8.96	0.60
Rhodamine G; C. I. 750	1.34	6.31
Fluorescein; C. I. 766 (color acid†)	0.03	2.21
" " " (sodium salt)	50.20	7.19
" " " (magnesium salt)	4.51	0.35
" " " (calcium salt)	1.13	0.41
" " " (barium salt)	6.54	0.56
Eosin; C. I. 768 (sodium salt)	44.20	2.18
" " " (magnesium salt)	1.43	0.28
" " " (calcium salt)	0.24	0.09
" " " (barium salt)	0.18	0.06
Erythrosin; C. I. 773 (sodium salt)	11.10	1.87
" " " (magnesium salt)	0.38	0.52
" " " (calcium salt)	0.15	0.35
" " " (barium salt)	0.17	0.04
Phloxine; C. I. 774 (sodium salt)	50.90*	9.02
" " " (magnesium salt)	20.84	29.10
" " " (calcium salt)	3.57	0.45
" " " (barium salt)	6.01	1.17
Rose Bengal; C. I. 779 (sodium salt)	36.25	7.53
" " " " (magnesium salt)	0.48	1.59
" " " " (calcium salt)	0.20	0.07
" " " " (barium salt)	0.17	0.05
Neutral Red; C. I. 825	5.64	2.45
Neutral Violet; C. I. 826	3.27	2.22
Iris Violet; C. I. 847	3.12	3.66
Nile Blue 2 B; C. I. 914	0.16	0.62
Metheylene Green; C. I. 924	1.46	0.12

*Gm. per hundred gm. of saturated solution.

†The color acids of the halogenated derivatives of fluorescein are practically insoluble in water.

THE MECHANISM OF STAINING
THE CASE FOR THE PHYSICAL THEORIES[1]

W. C. Holmes

(*Contribution No. 165 from Color and Farm Waste Division, Bureau of Chemistry and Soils, Department of Agriculture, Washington, D. C.*)

Abstract—**The application of the chemical, solution and adsorption theories to dyeing and staining processes is outlined briefly. It is held that adsorption is the principal factor in color retention.**

How should staining be visualized?

Before attempting to answer that question it will be well to inquire how practical dyeing is generally explained. Altho the aims and methods of textile dyeing, on the one hand, and of biological staining, on the other, often differ in a variety of respects, it is obvious that the operations are fundamentally analogous. The processes of dyeing, however, are much more readily susceptible to investigation than those of staining, and have received far more study. The conclusions of the chemist with reference to the nature of dyeing, accordingly, should be of considerable interest to the biologist.

It may be stated at once that in the past,[2] chemists have held very dissimilar views of the nature of dyeing, and that some divergence of opinion survives even today. It is probably safe to say, however, that the majority of chemists are now in general agreement respecting the nature of the factor which is most widely predominant in dye retention.

The three general theories which have been advanced to account for dyeing may be referred to as the chemical theory, the solution theory and the adsorption theory.

The chemical theory postulates a definite chemical reaction between the dye and the substance which is dyed, with the formation of a reaction product of definite molecular proportions. The color ion of the dye is usually assumed to combine with a salt-forming radical which is normally present in the substance that is being dyed, or is formed therein under practical dyeing conditions.

Altho it is somewhat difficult to comprehend how insoluble textile materials are able to enter into chemical reactions with dyes at all readily, and altho the ease with which dyes may often be extracted

[1]The case for the chemical theory is to be discussed in a later issue by another author.

[2]Wood, J. K. The Chemistry of Dyeing. London, 1913.

from dyed materials appears inconsistent with the conception of chemical union, the chemical theory of dyeing found extensive early acceptance. Its supporters were able to adduce a variety of evidence which they felt justified in interpreting in its favor. At a time when dyeing was practically confined to the application of the ordinary basic and acid dyes, and when cotton was necessarily dyed with the aid of mordants, it could be accepted as affording a possible, or even plausible, explanation of dyeing in general.

With the development of modern conceptions of physical chemistry, however, it has become evident that little, if any, of the evidence which has been advanced for the chemical theory has any decisive weight. Subsequent textile developments, moreover, have also shown that dyeing may be carried out readily when either, or both, the dye and the fiber are chemically inert. Altho the proponents of the chemical theory have waged a long and stubborn fight to uphold their views, the majority of chemists today attribute color retention in dyeing to causes other than definite chemical reactions.

The solution theory[3] postulates a passing of the dye molecule from solution in the dye bath to solution in the substance which is dyed thereby. The dye is held to distribute itself between the solution and fiber in the same manner in which it would between any two immiscible solvents in each of which it is soluble. Recent textile developments have served to revive the interest in this theory, which has, in general, found few adherents. A somewhat novel type of dye has been introduced for the coloring of artificial silk made from acetyl cellulose, and it is universally agreed among chemists who have investigated the question that the dyeing of acetyl cellulose with such dyes is a solution phenomenon.[4] It is generally held, however, that this type of dyeing is exceptional. Considerable evidence has been obtained which demonstrates that solution cannot be the factor of primary importance in dyeing processes in general.

The adsorption theory postulates a deposition of dye on the surface of the dyed material by adsorption.[5] The fiber is conceived of as adsorbing the dye in much the same manner as charcoal adsorbs the coloring matters of sugar solutions when employed in decolorizing them. No attempt will be made here to review in detail the evidence which supports such a view. It is sufficient to state that it appears to be well substantiated by the data of quantitative studies of dyeing reactions, and that it renders intelligible, in part or in whole, a variety of dyeing phenomena which appear inexplicable in the light of other hypotheses. Most chemists today believe that dyeing, in general,

---

[3]Witt, O. N. Färber-Zeitung, 11, 1890.

[4]Rose, R. E. Modern Theories of Dyeing. Am. Dyestuff Rep., 16, 14, 1927.

[5]Pelet-Jolivet, L. "Die Theorie des Färberprozesses." Dresden, 1910.

results primarily from adsorption rather than from other causes.

This concensus of opinion in respect to the nature of dyeing should have considerable weight with biologists. If adsorption is operative in the dyeing of wool and silk, which have well-defined chemical affinities and are dyed at elevated temperatures conducive to chemical reactivity, is it not reasonable to conclude that it is probably operative, also, in biological staining?

In reviewing typical staining phenomena, a variety of instances recur in which definite chemical reactivity appears extremely improbable. Fats are frequently stained, for example, by means of dyes which may be considered chemically inert. The bacteria of soils, again, are stained effectively by certain metallic salts of the dyes of the eosin group, whereas staining is ineffective with other metallic salts of the same dyes which would enter into chemical reactions with equal facility.[6] In these instances we may definitely reject the chemical theory and attribute staining to solution or to adsorption.

In other technics we find the biologist resorting to the employment of staining assistants of a type unfamiliar to the practical dyer. What may their function be? To cite a specific and typical instance, how may we explain color retention in the Ziehl-Neelson method for differentiating the tubercle organism in the diagnosis of tuberculosis?

The organism is stained with a solution of fuchsin containing phenol. It has recently been established that a definite addition product of the dye and phenol exists in such a solution[7] and constitutes the actual staining agent.[8] Neither the dye nor the phenol forms a stable chemical compound with the tubercle organism, and it is inconceivable that their addition product should do so. The addition product, however, may have (and would, in fact, be expected to have) colloidal characteristics decidedly different from those of the dye, which would enable the organism to adsorb it more readily and retain it more firmly.

A brief digression may be made here to inquire more particularly into the nature of the adsorption of dyes. In what form does their adsorption occur? Bancroft has recently declared that (in dyeing) basic and acid dyes are adsorbed in ionic form, whereas direct dyes are adsorbed as molecular aggregates.[9] This statement is coupled

[6]Conn, H. J. and Holmes, W. C. Certain Factors Influencing the Staining Properties of Fluorescein Derivatives. Stain Techn., 3, 94, 1928.

[7]Holmes, W. C. and Hann, R. M. Reactions of Basic Dyes with Cyclic Derivatives of an Acid Character. Stain Techn., 3, 122. 1928.

[8]French, R. W. Chemical Structure as Affecting the Staining and Therapeutic Properties of Dyes. Stain Techn., 1, 74. 1926.

[9]Bancroft, W. D. The Colloid Theory of Dyeing. Am. Dyestuff Rep., 18, 148. 1926.

with (and possibly conditioned upon) the further assertion that basic and acid dyes form true solutions, whereas direct dyes form colloidal solutions. Such sweeping generalizations appear unfortunate. It is well established that the aqueous solutions of basic and acid dyes are moderately colloidal in some instances and decidedly so in others.[10] Dyes in general are probably adsorbed in either or both the ionic and molecular form, depending upon attendant conditions.

The dual character of adsorption may be defined in broader terms. Both chemical and physical factors are operative. Adsorption is affected by the chemical nature of the adsorbing and adsorbed substances, and is also influenced by such physical factors as the surface area (and probably the density) of the adsorbing medium and the size of the adsorbed particle. In some instances chemical factors may be conceived of as predominating and in others physical factors.

The staining of the tubercle organism, previously referred to, may apparently be accepted as an instance of adsorption in which physical factors are predominant.

Turning to more complex phenomena, how may we visualize such instances of differential staining as occur with mixtures of dyes of the same general type? How is it possible, for example, for extra-nuclear elements to retain only methyl violet when stained with solutions in which another basic dye, methyl green (which is retained by chromatin elements), is the principal component? Such selective action appears decidedly inconsistent with accepted conceptions of chemical reactivity. It is not at all inconsistent, however, with the view of the dual nature of adsorption. Such differential staining may be accounted for by the hypothesis that chemical factors predominate in the adsorption of one dye by one element and physical factors in that of the other dye by the other element.

Similar considerations may be employed in accounting for metachromasy, in which it is very probable that the staining agents are tautomeric forms of the same dye.[11] Such tautomers may be assumed to have distinctly different chemical and physical characteristics which render them susceptible of adsorption of different types.

In general biologists resort to regressive types of staining technic. Specimens are overstained, and differentiation is obtained by washing out excess dye subsequently with such solvents as alcohol. May not the dual conception of adsorption account for such differentiation plausibly? Is it not reasonable to assume that decolorization would

[10]See footnote 4.

[11]Holmes, W. C. The Chemical Nature of Metachromasy. Stain Techn., 1, 116. 1926.

usually be easy and rapid when adsorption is of a primarily physical nature, and less readily effected when chemical affinities operate in a more marked degree? In any event it is difficult to reconcile the ease with which indifferent solvents such as alcohol remove dyes from stained specimens in general, with the view that any type of chemical compound is formed in the staining.

Differentiation is sometimes effected by substitution, wherein one dye which has stained tissue is discharged and replaced by a second dye. Analogous behavior is noted in adsorption processes.

In the field of vital staining it appears certain that the processes are those of adsorption. The colloidal characteristics of dyes which are suitable for vital staining lie within a comparatively narrow range. Other dyes with very similar chemical characteristics, but which yield either more highly aggregated, or less highly aggregated, solutions prove unsuitable.[12]

Controversy between the adherents to the chemical theory and to the adsorption theory of staining has centered in recent years about the staining of amphoteric substances. The Gram stain for bacteria is an instance in point, (and it may be suggested, in passing, that the essential function of iodine in the Gram technic is probably that of rendering the gentian violet more susceptible to adsorption by modifying its physical characteristics). The main issue under discussion may be put in a simple form. Why are bacteria, which are stained in neutral solutions only by basic dyes, stained only by acid dyes if the staining solutions are made decidedly acid?

The conventional explanation of the physical chemist is that the bacteria in question are negatively charged in neutral solutions and adsorb readily the positively charged basic dye particles. As the acidity of the solution is increased, however, they adsorb positively charged hydrogen ions in an increasing degree and basic dye adsorption diminishes. Eventually a point is reached beyond which the bacteria become negatively charged. At this point they cease to adsorb basic dyes and begin to adsorb acid dyes.

It appears probable, however, that such an explanation is incomplete, and that strictly physical factors also play a part in the staining. Basic dyes are converted from normal dye salts into polyacidic dye salts in the presence of considerable hydrogen-ion concentrations, and this transformation is attended by a decided alteration in the physical characteristics of their solutions. Solutions of basic dyes become less colloidal with increasing acidity. On the other hand acid dyes pass with increasing hydrogen-ion concentrations from normal dye salts to acid dye salts and to free color acids, and these substances are more colloidal than the normal acid dyes. Solutions

[12]Dyson, G. M. "The Chemistry of Chemotherapy." London, 1928.

of acid dyes become more colloidal with increasing acidity. In view of the degree in which the state of solute aggregation is known to influence adsorption in many instances it is evident that such alterations in the physical character of dye solutions may well exert, in themselves, an appreciable influence upon their staining behavior.

The proponents of the chemical theory argue that the bacterial substance in such instances is amphoteric and combines, or reacts, chemically with basic dyes above its isoelectric point and with acid dyes below that point.

Both arguments appear plausible, and decisive direct evidence on the point at issue is lacking, so far as the writer is aware. He feels, however, that the burden of proof rests with the proponents of the chemical theory in view of the weight of evidence that wool and silk are dyed by adsorption. The animal fibers are also amphoteric, and there is no reason for believing that their behavior toward dyes would differ in any essential particular from that of the bacteria under discussion.[13]

Admitting the possibility that chemical reactions may follow adsorption in some instances, and the possibility that solution may play a role of varying importance in numerous stainings, it is held that staining, in general, results primarily thru adsorption. Such a thesis is by no means novel. Its restatement herein is undertaken in the hope that it may serve in some degree to stimulate the active interest of the biologist in the further investigation of the questions at issue. It will probably prove extremely difficult to determine the nature of staining in various instances, but any attempts to do so are certain to yield much evidence on the influence of various factors upon staining which will be of great practical value in enabling the biologist to improve his technic and control its operation effectively.

---

[13]Since the foregoing paper was written recent comprehensive investigations of E. Yuri on bacterial staining, which afford considerable support for the general thesis advanced, have come to the attention of the author. ("The Studies on the Physical Affinity of Bacterial Cells to the Dyestuffs." Acta Scholae Medecinalis Universitatis Imperialis in Kioto, **11**, 75–115. 1928). Quantitative staining tests were made with more than 300 dyes on nine bacteria over an extensive pH-range and the quantitative aspects of the staining studied with about 60 acid dyes and a few basic dyes on two, or more, bacteria at several favorable H-ion concentrations. Yuri concludes that the general character of bacterial staining is one of adsorption in which electrostatic affinity plays the principal role. The data on basic dyes appear to indicate solution in some instances and adsorption in others, but the results were somewhat less decisive than with acid dyes, owing to interfering factors. With two acid dyes the phenomena appeared to be those of solution. With the great majority of acid dyes, however, the combination of dye and bacteria occurred in good accordance with the adsorption formula of Fruendlich.

DETAILED DIFFERENTIATION OF BACTERIA BY MEANS OF A MIXTURE OF ACID AND BASIC DYES AT DIFFERENT pH-VALUES

ALEXANDER V. TOLSTOOUHOV

Sydenham Hospital Laboratory, New York City

ABSTRACT.—**As previously reported by the author (1927), a mixture of methylene blue and eosin Y can be used for the differential staining of bacteria. It gives a fairly deep staining of bacteria at about pH 3 and above. Below pH 3 the eosin Y stains bacteria only a very pale pink; at such high H-ion concentration, the eosin is present as undissociated color acid, and for this reason not enough eosin is in solution to stain bacteria. To improve the staining at such reactions, the eosin was replaced by a stronger acid dye, namely acid fuchsin. The mixture of methylene blue medicinal Merck and acid fuchsin can be successfully used at a pH-value as low as 0.8. The method of staining by this new mixture is entirely the same as with the old mixture. It is sensitive enough to detect the difference in the isoelectric points: (1) of the single bacteria from the same pure culture, (2) of different strains of the colon and typhoid organisms. Some strains of the colon organism were found by this method with an isoelectric point at a pH-value as low as that of the Staphylococcus. Others, on the contrary, have their isoelectric point as high in the pH-scale as that of the typhoid organism. The new mixture can also be used for the study of the chemical composition of the different parts of bacterial body. Applying it at a definite pH-value, the author was able to stain differentially polar bodies of the typhoid group and of the diphtheria organism. This new mixture can be recommended in staining of *B. diphtheriae* as a substitute for Neisser's stain. It is interesting to note that polar bodies of the colon group consist of more alkaline protein than the body of the bacteria itself, i. e., they are stained by acid fuchsin. The polar bodies of the *B. diphtheriae* on the contrary are composed of more acid protein than the bacterial body; i. e., they are stained by methylene blue. The impossibility of detecting the above mentioned variations in the isoelectric points of bacteria using the Gram method is explained by the absence of pH variations in the latter technic. The differentiation of bacteria by the Gram stain depends chiefly on the varying stability of the compound formed (Gram-positive or Gram-negative bacteria plus gentian violet and iodine) in the presence of organic solvents, such as alcohol, acetone, etc.**

STAIN TECHNOLOGY, Vol. IV, No. 3, July, 1929

The idea of using a mixture of an acid and basic dye for differential staining of bacteria is not entirely new. E. W. Stearn and A. E. Stearn (1924) employed a mixture of carbol gentian violet and carbol acid fuchsin for this purpose. Lately, for some reason, they have abandoned this method and in their last article (1928) they advise using separately, carbol gentian violet or acid fuchsin, followed by flooding a smear with a buffer solution adjusted to different pH-values. Finally the smears are intensively decolorized. As a standard neutral decolorizer, they advise using acetone.[1]

While working with a mixture of methylene blue and eosin, it was found that the method for staining tissues and blood smears can be applied also for the differential staining of bacteria (Tolstoouhov, 1927). This mixture gives fairly deeply stained bacteria at about pH 3 and above. Below pH 3, eosin stains bacteria very poorly, a pale pink. From previous study it was learned that at such a low pH-value, eosin is present mostly in the form of undissociated acid, and for this reason cannot stain. To be able to use the mixture at a very low pH-value, eosin was replaced by acid fuchsin, which is fairly well dissociated under such conditions. As will be seen later, this is very important for those bacteria which are considered to have very acid isoelectric points.

Just as in preparing the mixture of methylene blue and eosin, there was experimentally found in this new mixture, a proper ratio and concentration of dyes at which their salt gave a maximum ionization. (See Tolstoouhov, 1927.)

Only under these definite conditions, was it possible to have deeply stained bacteria at a wide pH-range.

In the attempt to find a good combination of acid and basic dyes for bacteria, some other mixtures were tried, for example: Janus green plus acid fuchsin; crystal violet plus acid fuchsin; azure A or B plus acid fuchsin; safranin plus anilin blue; basic fuchsin plus anilin blue. The behavior of all these mixtures is about the same. The mixture of methylene blue plus acid fuchsin was preferred for the following reasons: 1) both of these dyes give practically no metachromatic effects over a wide pH-range (at a low pH-value, methylene blue has a slight greenish tint); 2) both methylene blue and acid fuchsin have pure colors, blue and red respectively.

For the purpose of examining the color of small objects, like bacteria, pure colors are very important factors. Only under these conditions, can there be sharp changes of color, at or near the iso-

[1]In their articles, the author could not find the exact composition of a dye solution and buffer mixtures.

electric point. It has been found that it is extremely hard to follow these color changes in the case of such mixtures as: crystal violet plus acid fuchsin; azure A or B plus acid fuchsin. Crystal violet or the azures have originally a deep violet tint, which is only slightly changed by the admixture of the red tint of acid fuchsin.

Method of Staining

Prepare separately in two bottles: 3.2% methylene blue, and 0.5% acid fuchsin[2] in pure methyl alcohol. Stock solution: 1.0 cc. of methylene blue 3.2% plus 10.0 cc. of acid fuchsin 0.5%. From

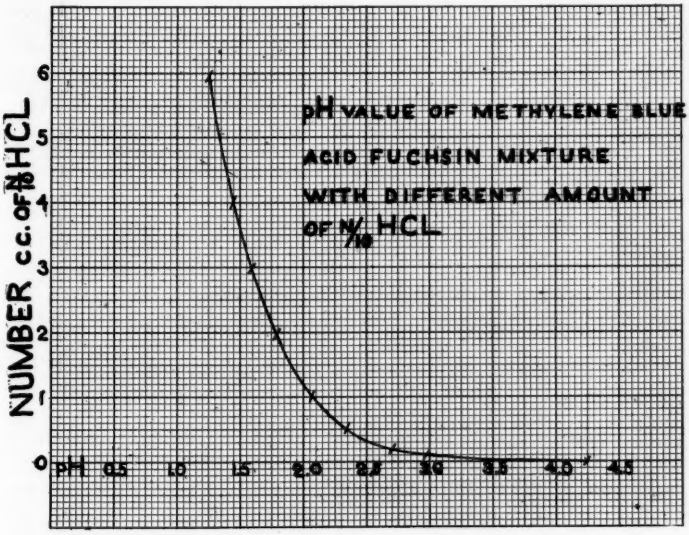

Chart No. 1. Reaction of the methylene-blue-acid-fuchsin mixture with different amounts of N/10 HCl in each tube of 10.0 cc.
Determination of pH-values were made with Biilman quinhydrone electrode.

this stock solution prepare the dilutions immediately before using. Into a series of test tubes, transfer increasing quantities of N/10 HCl beginning with 0, then 0.1 cc., 0.2 cc., 0.5 cc., etc. In case a lower pH-value than 1.4 is necessary, take N/1 HCl instead of N/10, in the following series: 0.7 cc., 1.0 cc., 2.0 cc., 3.0 cc. To each tube add 1.0 cc. of the dye stock solution and water to make the total volume 10.0 cc. The dye mixture adjusted to the different

[2]Merck's medicinal methylene blue, and Grübler's acid fuchsin (purchased about two years ago) were used in the present work.

reactions is not stable and should be prepared immediately before using. The pH-value of each test tube can be found on Charts No. 1 and 2. The pH-values on these charts were found electrometrically, using a quinhydrone electrode. (Biilman, 1924).

Preparation of Slides. Prepare a suspension of 1 or 2 loops of bacteria under examination with 3 or 4 drops of distilled water

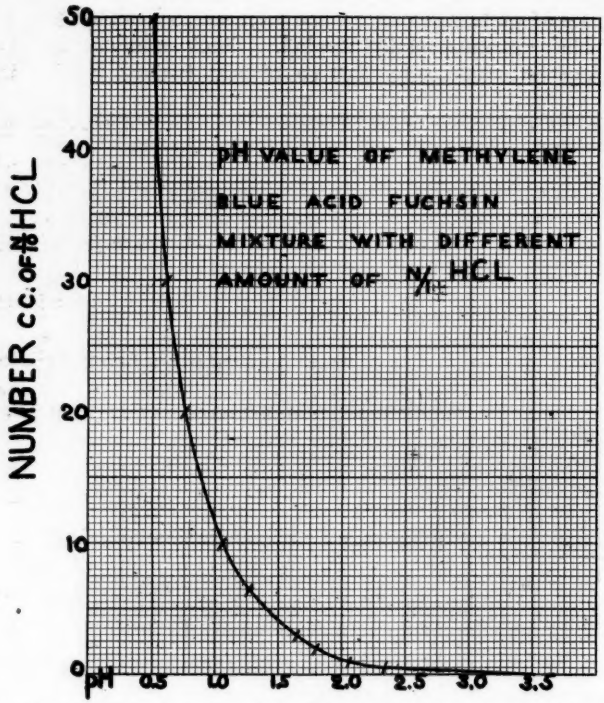

Chart No. 2. Reaction of methylene-blue-acid-fuchsin mixture with different amounts of N/1 HCl in each test tube of 10.0 cc.

For uniformity with Chart No. 1 the ordinates on this chart are expressed in terms of N/10 HCl instead of actual N/1 HCl.

Determinations of pH-values were made with Biilman quinhydrone electrode.

placed on a microscopic slide. Transfer one loop of the suspension on a series of slides. Let them dry in the air and then pass as usual 3 or 4 times thru the flame.[3]

Staining. Cover a series of slides with the dye mixture adjusted respectively to the series of pH-values for 2 or 3 minutes. Rinse

[3]Forced drying over a flame can change the bacterial proteins.

with distilled water. Blot with filter paper and examine under the microscope.[4]

Experiment

For this experiment the following bacteria were taken: *Bacterium typhosum* (culture received from New York Board of Health);

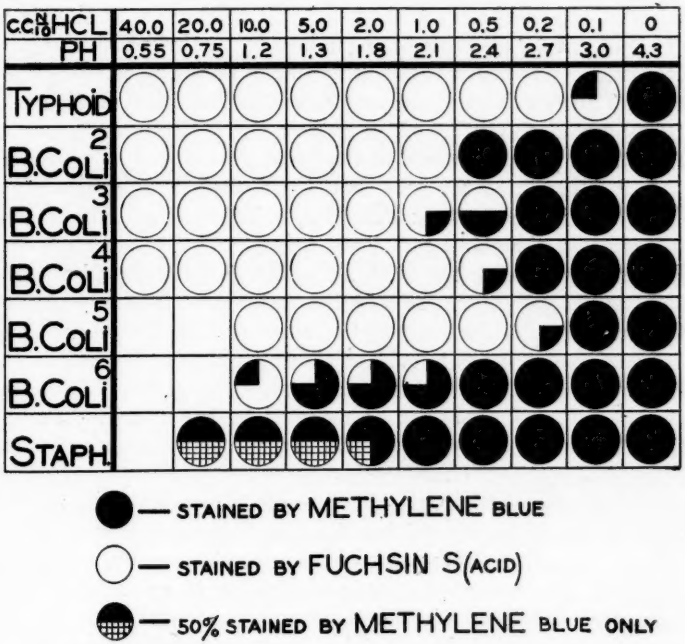

Chart No. 3. Results of staining different bacteria with the methylene-blue-acid-fuchsin mixture at different pH-values.

Note that different bacteria begin to be stained by acid fuchsin at different points. *B. typhosum* is assumed to be composed of a more alkaline ampholyte, with its isoelectric point at about pH 3.5. Among the different strains of *B. coli* are seen some which are composed of ampholyte with an isoelectric point slightly more acid than that of *B. typhosum*, as No. 5. Others, as No. 6, are composed of a much more acid ampholyte, with its isoelectric point almost as low in the pH-scale as that of a Staphylococcus.

5 strains of *B. coli* (cultivated from genito-urinary tract of the patients from Sydenham Hospital) and *Staphylococcus albus* (cultivated from a wound). The identification of the cultures was made in the

[4]The distilled water can be omitted. Immediately after staining, blot with filter paper.

usual way by subculturing on the special media and by Gram staining, and following the description under *Bacillus coli communis* in Zinsser and Tyzzer's "A Textbook of Bacteriology, *6th ed.* 1927, pp. 533, 539–541.

All the above mentioned bacteria except *Staphylococcus albus* were definitely Gram-negative. The results of staining with the writer's mixture are presented on Chart No. 3. As will be seen by this chart, at about pH 4.3 all bacteria are stained by methylene blue in pure blue color. At about pH 3.0, *B. typhosum* begins to be stained red by acid fuchsin. In accordance with the definition of Michaelis (1926) and on the theory of the Stearns (1924, 1928) and the writer (1927), this pH-value would be the isoelectric point of *B. typhosum.*[5]

As will be seen by the same chart No. 3, the various strains of the colon organism begin to be stained by acid fuchsin at different pH-values or on the above mentioned theory, they have the different isoelectric points. Some of them, as No. 5, have their isoelectric point at pH 2.6. They are only slightly different from *B. typhosum.* Others, as No. 6, are composed of more acid ampholytes with an isoelectric point at pH 1.25. The above mentioned difference in the isoelectric points (or in other words, staining properties) of different strains of *B. coli*, could not be detected by means of the routine Gram method. The reason for that is simple—the absence of variations of pH-value in Gram method.

Differentiation of bacteria by means of the classical Gram method depends chiefly on the varying stability of the compound formed (Gram-positive or Gram-negative bacteria plus gentian violet and iodine) in the presence of organic solvents, such as alcohol, acetone, mixture of equal parts acetone and alcohol, or mixture of equal parts isopropyl and methyl alcohol. The above mentioned compound with Gram-negative bacteria is less stable in the presence of these solvents, and for this reason, as we say, Gram-negative bacteria are decolorized in Gram staining.

Just as in the author's previous method of tissue staining all interfering factors were excluded from the technic of bacterial staining, namely: decolorizers, salt effect, mordants and commonly used compounds such as anilin oil or phenol.

In order to get a definite pH, a certain very small amount of HCl was added directly to the dye mixture. The smears were not flooded with buffer mixtures after previously staining them. This flooding of the smear with buffer mixture introduces two uncontrollable

[5]This means the isoelectric point of the ampholyte, nucleoprotein or phosphatide, (see Stearn and Stearn 1928), composing the body of typhoid bacillus.

factors: 1) salt effect, as all buffer mixtures known to the author contain solutions of electrolytes much more concentrated than the dye solutions; 2) it makes the dye concentration unknown, very dilute and variable.

As will be seen later, the stability of the salt (some dye and bacterial ampholyte) is different with Gram-positive and with Gram-negative bacteria. For this reason, in order to study the isoelectric points of the bacterial ampholyte but not the varying stability of the salt (dye plus bacterial ampholyte), the same comparatively strong dye concentration was used in every test tube. It means that every smear is always covered with a mixture which has only one variable, namely pH-value.

The use of any kind of decolorizers in staining introduces, besides the one variable, (pH) another variable, namely, the stability of the salt (dye bacterial ampholyte) in the presence of an organic solvent. It is natural that in studying the isoelectric points of bacterial ampholyte, decolorizers were discarded entirely. Besides it is agreed that decolorizers very often are not only solvents but like the dyes they form compounds with proteins and phosphatides. It is known that compounds like phenol, aniline, or other mordants, change the staining properties of the ampholyte. The effects of phenol and aniline oil were studied by the Stearns (1928). The effect of some mordants on the isoelectric point of the tissue protein was studied by the author (1928).

It is now clear from a chemical standpoint that the use of these buffers, mordants, decolorizers, etc., makes the interpretations of the staining results more complicated and uncertain. For that matter, the author does not understand why the above mentioned complications were introduced in the technic of staining.

The Polar Bodies of the Colon Group and the Variation in the Isoelectric Points of a Single Bacterium from the Same Pure Culture

The sensitiveness of the method of staining described above, is evident from the following facts:

It has been found invariably that the individual bacteria from the same pure culture are apparently composed of ampholytes with slightly different isoelectric points.[6] In other words, at a definite pH, some bacteria are red, some blue.

It should be emphasized that on Chart No. 3, the color of a majority of the bacteria at a given pH-value is marked and not the

[6]Subcultures, used for staining were not more than 14 hours old. Very often subcultures 7 to 9 hours old were used.

color of a single bacterium; it means that the determination of the average isoelectric point of the whole culture of a micro-organism has been made, and not that of a single representative of it.

In practice, it is done in the following way: It is preferable to take for examination, comparatively heavy parts of the smear where the whole field of the microscope is packed with bacteria. The analysis is started at the higher pH-value at which all bacteria are blue. In examining the smears, the color of each single organism was not examined, but the color of the whole field. In this way, the smears on which the majority of bacteria have changed from blue to purple or red, are easily noticed. This point is marked as the isoelectric point of that particular culture. It is much easier to see the change of color from blue to purple or red than vice versa and, therefore, this analysis was started with the smears stained at the highest pH-value.

It seems that the most natural explanation for the above mentioned variation in the isoelectric points of the single bacterium is that the individuals are of different ages.

The above described method reveals also the presence of polar bodies in the bacteria of the colon group. These polar bodies are seen on the smears stained at pH 2.7–2.1 as red dots on the ends of the bacteria, while the bacterial body is stained bluish purple. At pH 2.1 they are stained more purple or purple red. The isoelectric point of these polar bodies is, therefore, assumed to be at about pH 2.4.

Staining of *Bacillus Diphtheriae*

To study *B. diphtheriae* staining, smears from nose and throat cultures prepared on Loeffler's media were used.

Method of staining

The same method was used as for the colon group described above. Beginning at pH 4.3 up to pH 1.2 the polar bodies of the *B. diphtheriae* are very deep blue, the bacterial body itself a light blue. At pH 1.2 and lower, they are stained a much lighter blue and the bacterial body turns light purple blue. The other bacteria are red. At pH 4.3–2.1 the polar bodies are very deep blue. At this reaction they are stained much deeper and are more distinctly visible than on a smear stained by the Neisser method. For routine work, the author advises using methylene blue 0.2% solution mixed with HCl at pH 4.0–3.0.

No doubt a study of the staining properties of *B. diphtheriae* similar to the study of the colon group described above would reveal more subgroups in *B. diphtheriae*.

As will be seen by our future reports, the existence of *B. coli* with different isoelectric points will explain the variable results of the *B. coli* infection treatment of the genito-urinary tract.

Conclusions

The results of the proposed method of bacteria staining by the mixture of acid plus basic dyes at various pH reveals:

(1) The presence among the colon group of variants with different isoelectric points. Some of these bacteria have the isoelectric point at a pH-value almost as low as the Staphylococcus. The other ones on the contrary, have the isoelectric point as high as the isoelectric point of *B. typhosum*.

(2) The variations in the isoelectric points of the single bacteria from the pure culture. These variations probably depend on the age of the different single bacteria.

(3) The presence in *B. coli* of polar bodies.

(4) The same method can be used for staining *B. diphtheriae*. As revealed by this method the polar bodies of *B. diphtheriae* are composed of an ampholyte with an isoelectric point at the very low pH-value, while on the contrary, that of the polar bodies of the colon group have a very high pH-value. The principles involved in differentiation of bacteria by the Gram method and by the mixture of acid and basic dyes at various pH-values are to be found in the main article. The use of adjuvants such as decolorizers, buffer mixtures, phenol, etc., in staining, is also described above.

BIBLIOGRAPHY

Biilman, Einar. 1924. Oxidation and reduction potentials of organic compounds. Trans. Far. Soc., 19, 676.

Conn, H. J. 1928. A new substitute for ethyl alcohol in the Gram stain. Stain Techn., 3, 71.

Kopeloff, N., and Cohen, P. 1928. Further studies on a modification of the Gram stain. Stain Techn., 3, 64.

Michaelis, L. 1926. Hydrogen ion concentration. Translation by Perlzweig. Williams & Wilkins Company. Baltimore. See Vol. 1, 64.

Stearn, E. W., and Stearn, A. E. 1924. The chemical mechanism of bacterial behavior. I. Behavior toward dyes—factors controlling the Gram reaction. J. Bact., 9, 463.

1928. The effect of the chemical nature of a decolorizer on its functioning. Stain Techn., 3, 81–87.

Tolstoouhov, A. V. 1927. Some practical applications of the physico-chemical theory of differential staining. Blood, tissue and bacteria staining. Methylene blue, eosin water soluble mixture as an universal dye mixture. Proc. N. Y. Path. Soc., N. S., 26, 147–159.

1928. The effect of preliminary treatment (fixing fluids) on staining properties of the tissues. Stain Techn., 3, 49.

A NOTE ON THE GRAM STAIN

ALEXANDER ZEISSIG, *Dept. of Pathology and Bacteriology, N. Y. State Veterinary College, Ithaca, N. Y.*

ABSTRACT.—**A modification of the Gram stain in which iodine-alcohol is substituted for 95% alcohol as a decolorizing agent has been found particularly useful in staining Gram-positive organisms in tissues and also for smears. The technic for tissue sections follows:**

1. **Apply nuclear stain.**
2. **Wash.**
3. **Stain in Hucker's gentian violet 2 to 3 minutes (i. e. 1 part Sat. Alc. Sol. crystal violet to 4 parts 1% Aqu. Sol. ammonium oxalate).**
4. **Wash in water.**
5. **Stain in Gram's iodine 5 minutes.**
6. **Wash in water.**
7. **Decolorize in 95% alcohol to which enough tincture of iodine has been added to give a mahogany color.**
8. **Counterstain.**
9. **Dehydrate and mount.**

While staining tissue sections in order to study the relationship of Gram-positive organisms to the lesions they produced we experienced considerable difficulty in obtaining sharp differentiation of the organisms and the host tissues. Muscle and connective tissue especially held the gentian violet very tenaciously and in order to remove the dye completely from these tissues it was necessary to decolorize a considerable length of time in 95% alcohol. This prolonged treatment also resulted in the complete removal of the gentian violet from the invading organisms.

It is known that treatment of organisms previously stained with gentian violet results in the fixation of the stain in Gram-positive organisms so that they will resist subsequent decolorization with 95% alcohol for a time at least, whereas Gram-negative organisms similarly treated are promptly decolorized. We reasoned that by keeping the organisms in constant contact with iodine during the decolorizing process the selective fixation of the gentian violet by the iodine would be continued, while at the same time the decolorization of Gram-negative organisms and tissue elements would proceed as usual. Enough tincture of iodine was therefore mixed with the decolorizing alcohol to give it a deep brown color, the tincture being selected so as to maintain the concentration of alcohol. The sections

were treated as usual, being stained in Hucker's gentian violet[1] followed by Gram's iodine. They were then decolorized in the alcohol-iodine solution. On trial the desired result was obtained. The prolonged exposure to alcohol made possible by the presence of the iodine which maintained its selective fixing action on the Gram-positive organisms, resulted in very sharp differentiation of the organisms and the host tissues.

This same procedure has been applied to bacterial smears. It is particularly useful when the smears are made from lesions and contain tissue elements, or with such substances as milk. Here the elements of the smear other than the organisms are stained with gentian violet which is difficult to remove without decolorizing the organisms also. By decolorizing in alcohol-iodine these elements are decolorized and take the counterstain resulting in sharp differentiation. The use of this modification of the decolorizer will also be of advantage in staining smears made from pure cultures, since the time element in the decolorizing process will not be so important. While it will not eliminate Gram-variable organisms, it will, especially with beginners, be of advantage since a little over-decolorization will not be so apt to result in an organism commonly described as Gram-positive appearing Gram-negative as when plain alcohol is used to decolorize smears. In classroom work we have found this procedure to be quite satisfactory in staining bacterial smears.

---

[1]The dye employed in this formula has ordinarily been certified crystal violet obtained from the National Aniline and Chemical Co. The formula followed was one part saturated alcoholic solution crystal violet to four parts of a 1% aqueous solution of ammonium oxalate.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order them direct from STAIN TECHNOLOGY, at a price of 6 cents each.

FRY, H. J. A paraffin method for serially sectioning a minute object in a known plane. Anat. Rec., 34, 245–252. 1927.

A method is described for the handling of minute objects during the process of fixation, dehydration, clearing and embedding. It is claimed that the method is simple, prevents loss or damage of the objects, and permits embedding in a known plane. A device for filtering the wash water is described. It is recommended that all reagents used be filtered, that the reagents be changed by means of a lip pipette, and that the object remain in the small round bottomed vial in which it is fixed until it is ready for embedding. At this time it is changed to a small white porcelain embedding dish, such as paints are sold in, by use of the lip pipette. The xylol is then replaced with paraffin and the object is oriented as desired. For further details the original article should be consulted.

KISSER, J. and ANDERSON, B. B. Method of preparing thin cross and longitudinal sections of cotton fibers and its importance in cell wall research. Am. J. Bot., 15, 437. 1928.

Stain fibers for a few hours in carmalum so that they will be visible after they are embedded. Wash and dry on filter paper. Stretch the fibers on a cardboard frame by fastening first one end and then the other with a thick solution of gum arabic. Place the frame in 96% alcohol for 24 hours and then for 24 hours each in 2, 4, and 8% celloidin in a small embedding dish covered with a glass plate. When the celloidin is of the proper degree of hardness place the block in chloroform for a few hours for further hardening; then remove, cut out the center of the frame with a very sharp knife, and replace the block in chloroform until it is saturated. Add paraffin of 52° melting point and place in the oven; embed the block in the usual way. Cut the sections with a sliding microtome with the knife edge cutting at an angle of 15° and moistening the blade with gelatin water to keep the sections from sticking to the knife. Cross sections may be cut 2μ thick and longitudinal ones 4μ. Sections are fixed to the slide as usual with albumen fixative, paraffin and celloidin are removed from the sections with xylol and ether respectively. Permanent mounts may be made with glycerin or glycerin gelatin. Thin sections prepared in this way may be used for X-ray studies of the cell wall.

SWEANY, H. C. System of sputum analysis for the presence of acid-fast bacilli. J. Lab. & Clin. Med., 14, 547–557. 1929.

The system presented is the one in use at the Chicago Municipal Tuberculosis Sanatorium and deals with new and effective concentration method. Specimens are incubated 18 to 24 hours. Upon removal from the incubator the specimens are treated with an equal amount of 3% NaOH thus making the concentration of NaOH 1½%. Bottles are next shaken for 15 to 20 minutes in a shaking machine, incubated for 30 minutes and then centrifuged at 3000 r.p.m. for 8 minutes. The supernatant liquid is poured off and the top of the sediment is scooped off with a platinum loop and is smeared on a slide. Slides are placed in a rack fixed over the electric bath and are stained 8 to 10 minutes by Cooper's method. They are then examined for 6 minutes. By this method the sputum is concentrated more than 50 times and the method approaches the accuracy of animal inoculation in early diagnosis. Experiments were carried out testing the efficacy of incubation and relative efficiency of the method reported and methods using 3% HCl, 1–2 and 3% NaOH, and the antiformin method.

TERRY, B. T. Improvement in technic and results made in examining microscopically by the razor section method 2000 malignant tissues. J. Lab. & Clin. Med., 14, 519. 1929.

Improvements in the original technic described by the author make it possible to cut thin slices of tissue still thinner; to section very small bits of tissue and to obtain good results with refractory tissues such as those containing large amounts of colloid or mucus or tissues that are so soft that they make sectioning difficult. Fixation in hot formalin makes it possible to use the author's method on tissues of the above type. Staining with Harris' hematoxylin is recommended in addition to the usual neutralized polychrome methylene blue staining. This enables those who are accustomed to hematoxylin staining to use the razor section method. Diagnosis made by this method was found to check with diagnosis made by the Mayo clinic pathologists using the frozen section method in 98% of the 2000 cases. The original technic together with the suggested improvements is described in detail.

BELLING, J. A method for the study of chromosomes in pollen-mother-cells. Univ. of Cal. Publs. in Bot., 14, 293–299. 1928.

Chromic-acetic-formaldehyde (1:10:16 or 1:10:8) is recommended as a fixative in place of the expensive Flemming's solution. Iron-brazilin is recommended to replace iron-hematoxylin as a stain. The smear method is recommended in place of the paraffin method. Excellent preparations of the leptophase, zygophase, pachyphase, and diplophase are obtained by this method. For details of the technic the original article should be consulted.

CARTWRIGHT, K. ST. G. A satisfactory method of staining fungal mycelium in wood sections. Ann. Bot., 43, 412. 1929.

Stain section with 1% aqueous safranin for a few seconds. Wash with water. Stain in picro-aniline blue made up by adding 100 cc. saturated aqueous picric acid to 25 cc. of saturated aqueous anilin blue. (No exact information as to nature or source of dyes is given.) Cover the section with the stain, and hold over the flame until the solution reaches the simmering point. Wash out all the blue which comes out easily, then wash in changes of 95% alcohol and in absolute alcohol. Clear in clove oil and mount in Canada balsam. By this technic the lignified walls will be stained red and the fungus mycelium a clear blue. The finest hyphae may be easily traced thru the wood.

DOLFINI, G. Su un nuovo metodo di colorazione dei grassi. Bull. d'Histol. Appl., 6, 137. 1929.

Using the method of Galesesco and Bratiano abstracted in STAIN TECHNOLOGY 4, p. 52, the authors conclude that this method of staining fat with alcoholic extract of carrots (Daucus carota) is inferior to fat staining with Sudan and that it is useless in the identification of different kinds of fat.

DOUBROW, S. Un procédé rapide de coloration des bacilles de Koch dans les coupes histologiques. Bull. d'Histol. Appl., 6, 142. 1929.

The method given is a combination of the method of Much of differentiation with picric alcohol and that of Masson for staining nuclei with iron-hematoxylin. Paraffin sections 5μ thick are treated with collodion by Regaud's method, are mordanted fifteen minutes in a 5% iron-alum solution at 50°. Wash quickly in distilled water. Treat for 15 minutes with Regaud's hematoxylin at 50°. Wash quickly in tap water. Stain with Ziel's solution at 50° for 45 minutes (exact formula employed not given). Differentiate with picric alcohol (saturated solution in 95% alcohol diluted one-half) until only the nuclei appear stained under the low power. Wash in 95% alcohol, mount in balsam. Koch bacilli are red, nuclei black, back-ground yellow or orange. The picric acid may be washed out with water and the background colored with light green as the Koch bacilli stand out better against this background. The method has the advantage of speed and differentiation of the nuclei and the bacilli with the same reagent.

HADJIOLOFF, A. Coloration des graisses par quelques pigments naturels. Bull. d'Histol. Appl., 5, 183. 1929.

Tests were made of the pigments of pepper, tomato, carrot, pumpkin, orange rind, beets, red cabbage, cherry, and grape as fat stains. The extracts were made by

putting the previously dried fruits or vegetables in 85% alcohol and allowing them to stand for several days at 37°; this solution is filtered twice and used for staining fat in adipose tissue. Tomato, pepper, and pumpkin gave the best results, giving a staining like Sudan. Carrot was not so good, and the pigment of beets, red cabbage, cherries and grapes gave no fat staining.

JOYET-LAVERGNE, PH. La recherche qualitative du glutathion. Bull. d'Histol. Prac., 5, 331–349.

The methods given in this paper are the same as already reported from a different source. (See STAIN TECHN., 4, 29.)

LEVINE, M. A method for staining connective tissue mast cells. J. Lab. & Clin. Med., 14, 172. 1928.

1. Fix material in any of the usual killing agents.
2. Embed in paraffin.
3. Section material 5 to 10 μ.
4. Remove paraffin in xylol.
5. Remove xylol in 95% alcohol.
6. Rinse in water.
7. Stain in 1 to 2% thionin in water. (Information as to source or dye content of stains not given.)
8. Remove excess stain in water, remove water about sections, dehydrate.
9. Stain in solution of orange G in oil of cloves.
10. Observe preparation under microscope until desired results are obtained.
11. Wash thoroly in xylol.
12. Mount in Canada balsam.

Cytoplasm stains faint orange. Mast cells stand out stained deep blue or purple color and the difference between these and stroma nuclei is well marked by difference in intensity of staining, the latter staining faint blue. Material used for staining in these experiments was parathyroid glands of steer, cow, rat, mouse, cat, rabbit, and man.

MARTENS, P. Les structures nucléaires et chromosomiques dans la cellule vivante et dans la cellule fixée. Bull. d'Histol. Appl., 5, 229–252. 1928.

Observation of the resting nucleus of the living cell made it apparent that the nucleus of this cell is made up of a reticular filamentous structure, with granular embedding at the intersections of the network. A second method of studying the problem was the close observation of the cell during the process of fixation. Bouin and Benda's fluids were most used and of these Benda's was most favored by the author. With this fluid, fixation occurs rapidly but not too strenuously. The only result of fixation is that visibility of the existing structure is accentuated and no new structure is created by the process of fixation.

PARKER, F. P. A technic for staining red blood cells for measurement of cell diameters by the projection method. J. Lab. & Clin. Med., 14, 663. 1929.

After the blood smear is fixed by heat, stain with a saturated solution of eosin in 95% alcohol for two minutes, wash with distilled water, stain with 1% aqueous solution of methyl violet for two minutes, wash with distilled water and dry in the flame. (No definite information as to nature or manufacture of dyes employed is given.) The red cells are stained a deep bluish violet and contrast well with the background, thus insuring more accurate measurements.

PARKER, F. P. and LEWIS, G. T. Microscopic projections in the measurements of erythrocytes. J. Lab. & Clin. Med., 14, 664. 1929.

A projection method for measuring erythrocytes is presented accurate to 0.25 microns. A 400 watt flood light with concave reflector and planoconvex lens three inches in diameter is used as the source of illumination. For magnification a monocular microscope with the condenser, mirror, and draw tube removed is used. With a 1.8 mm. oil immersion objective the magnification that may be secured with the apparatus is five hundred diameters.

PERGOLA, M. Substrati al tellurito e colorazione ridotta nella ricerca e dimostrazione del bacillo difterico. Bull. dell' Ist. Sieroterapico Milanese, 7, 585–598. 1928.

The writer revises his method of culture for the diphtheria organism on serum-egg-tellurite medium (abbreviated S. U. T.), his modification containing glycerinated yolk with potassium tellurite. His method of reduced staining is as follows:

1. Stain 10 to 15 seconds or more with Neisser's blue; prepared as follows:
   Methylene blue (Grübler) ..... 1 g.
   Ethyl alcohol (96%) ..... 20 cc.
   Dissolve and add:
   Glacial acetic acid ..... 50 cc.
   Distilled water ..... 950 cc.
2. Wash with distilled water.
3. Treat with Lugol's iodine solution 10 to 15 seconds or more.
4. Wash with distilled water.
5. Finally stain with chrysoidin solution (1:3000) for a few seconds. (Further information as to the nature of this dye not given.)

By this technic the Babes-Ernst polar or metachromatic granules are black or dark blue, the bacterial bodies light blue.—*R. Ciferri.*

VOLKONSKY, M. Sur une nouvelle modification de la technique d'Altman. Bull. d'Histol. Appl., 5, 220–222. 1928.

Stain in 20% rubin S in anilin water on the hot plate. Wash in distilled water. Differentiate in aurantia, 5% solution in 70% alcohol, until only the mitochondria remain red. For rapid examination mount in glycerin and then wash off quickly. Treat 3 or 4 minutes with the following mixture: Phosphomolybdic acid, 1 g.; normal soda, 10 cc.; distilled water, 90 cc. Wash carefully and stain 10 to 15 minutes in the following mixture: methylene violet, 0.4 g.; methylene azure II, 0.1 g.; potassium carbonate, 0.1 g.; glycerin, 50 cc.; distilled water, 50 cc. (Further information as to nature or dye content of stains not given.) Wash in water, differentiate in Unna's tannin-orange. Wash, dehydrate and mount. Mitochondria are red, nuclei blue or violet, cytoplasmic inclusions yellow or orange. The best fixative for this staining method is Deitrich-Parat which is Helley's fixative followed by treatment with chromium salt for 48 hours at 37°. Meves' and Regaud's fixatives also give good results.

WEBBER, J. M. Smear method for the study of chromosomes. Univ. of Cal. Publs. in Bot., 14, 345–352. 1929.

Fix in 10 cc. glacial acetic+25 cc. absolute alcohol 10 minutes.
Wash in three absolute alcohols 5 minutes each.
95, 80, 70, 60% alcohol 5 minutes each.

Stain 1:
Delafields' hematoxylin—8 to 10 minutes. Rinse in 60% alcohol.
Differentiate in acid alcohol (5 drops HCl to 40 cc. 70% alc.)
Rinse 60% alc., then 70, 80, 95% and absolute 5 minutes each.
Clove oil—15 seconds, 80% xylol few seconds, xylol 8 to 10 minutes.
Canada balsam.

Stain 2:
Iron alum (20 cc. abs. alc.+15 cc. 4% aqu. iron alum) 1 hour.
Rinse in 60% alc. Wash three changes 70% alc. 8 minutes each.
Brazilin (0.5% in 70% alc.) 1 hour.
70, 80, 95%, and abs. alc. 5 minutes each.
Destain in 70% alc. Clear cytoplasm in iron alum.
Dip several times in 70 and 95% alc. Two changes abs. alc. 5 minutes each. Flood with clove or bergamot oil, then 80% xylol. Five minutes each in 2 xylols. Hydrax or Canada Balsam.

The method is good for the study of matured chromosomes, cytokinesis, tetrad cells, etc.; but for finer details of the thread stages the author suggests Bellings' brazilin method.